Mutational analysis of whole mitochondrial DNA in patients with MELAS and MERRF diseases

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Abstract

Mitochondrial diseases are clinically and genetically heterogeneous disorders, which make the exact diagnosis and classification difficult. The purpose of this study was to identify pathogenic mtDNA mutations in 61 Korean unrelated families (or isolated patients) with MELAS or MERRF. In particular, the mtDNA sequences were completely determined for 49 patients. From the mutational analysis of mtDNA obtained from blood, 5 confirmed pathogenic mutations were identified in 17 families, and 4 unreported pathogenically suspected mutations were identified in 4 families. The m.3243A > G in the *tRNA^Leu(UUR)* was predominantly observed in 10 MELAS families, and followed by m.8344A > G in the *tRNA^Lys* of 4 MERRF families. Most pathogenic mutations showed heteroplasmy, and the rates were considerably different within the familial members. Patients with a higher rate of mutations showed a tendency of having more severe clinical phenotypes, but not in all cases. This study will be helpful for the molecular diagnosis of mitochondrial diseases, as well as establishment of mtDNA database in Koreans.

Keywords: DNA, mitochondrial; MELAS syndrome; MERRF syndrome; point mutation

Introduction

Mitochondrial diseases are clinically and genetically a very heterogeneous disorder group. Some mitochondrial disorders only affect a single organ, such as the eye in Leber hereditary optic neuropathy (LHON), but most diseases involve multiple organ systems and often typically manifest in tissues with high-energy demand, e.g., nerve and muscle. The multi-organ involvement and overlapping of symptoms among mitochondrial disorders make the exact diagnosis and classification difficult. Identifying mitochondrial DNA (mtDNA) mutations is now an important method in the diagnosis of patients with mitochondrial disorders. The mtDNA mutations are largely divided into two groups. The first group is comprised of point mutations in tRNA, rRNA, or protein coding genes, which are commonly maternal inheritance. The second group is composed of the rearrangements of mtDNA, such as duplication or large deletion, which are usually either maternally inherited or sporadic (Schmiedel et al., 2003). The frequent mitochondrial disorders caused by the point mutations of mtDNA are mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes (MELAS, MIM# 540000), and myclonus epilepsy with ragged-red fibers (MERRF, MIM# 545000).

To date, several hundreds of different mtDNA mutations have been reported to be associated with various mitochondrial diseases in a Human Mitochondrial Genome Database (MITOMAP: http://www.mitomap.org) (Ruiz-Pesini et al., 2007). Of them, the m.3243A > G, m.3271T > C, m.3291T > C and m.10191T > C in MELAS, and m.8344A > G, m.8356T > C and m.8363G > A in MERRF are well-confirmed common point mutations (Goto et
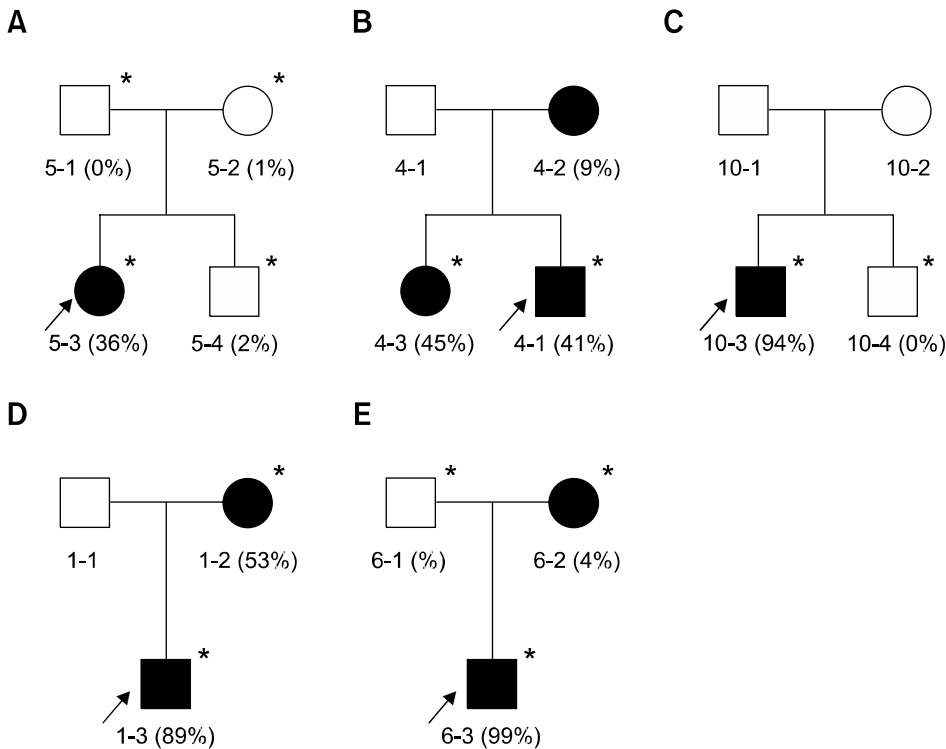
Figure 1. Pedigree analysis and variable heteroplasmic rates among family members. Each family member showed very different heteroplasmic rates in most pedigrees. The available DNAs are indicated by asterisks (*). The filled (■, ●) and open symbols (□, ○) represent affected and unaffected members, respectively. The arrows indicate probands. (A) MT5 MELAS family with m.3243A > G, (B) MT4 MELAS family with m.3271T > C, (C) MT10 MELAS family with m.10191T > C, (D) MT1 MERRF family with m.8344A > G, and (E) MT6 MERRF/PEO overlapping family with m.8344A > G.

al., 1990, 1991, 1994; Shoffner *et al.*, 1990; Silvestri *et al.*, 1992; Santorelli *et al.*, 1996; Taylor *et al.*, 2001). Many MELAS and MERRF patients have shown one of these mtDNA mutations, however, no causative mutation has still been identified in a large part of patients.

The mtDNA mutational studies have not been actively performed in Korean patients (Kwon *et al.*, 2004; Choi *et al.*, 2008). Moreover, complete mtDNA sequencing data for Koreans are very limited. In the present study, the mtDNA sequence was analyzed to find the pathogenic mutations in 61 families that have MELAS or MERRF in Korea. In particular, the mtDNA sequences were completely determined for 49 patients. In our study, 9 mutations (5 confirmed and 4 suspected) were identified in 21 patients, and the correlation between the heteroplasmic rates and the severity of clinical phenotypes were conducted.

Results

Identification of causative mutations

Five reported pathogenic point mutations were identified in 17 families from the screening of 61 families or isolated patients (27.4%). The m.3243A > G in the *tRNA^{Leu(UUR)}* was observed in 10 MELAS patients, followed by m.8344A > G in the *tRNA^{Lys}* of 4 MERRF patients. Others were

m.3271T > C in the *tRNA^{Leu(UUR)}*, and m.10191T > C (Ser45Pro) in the ND3 of each MELAS patient, and m.8363G > A in the *tRNA^{Lys}* of a MERRF patient. Details of the mutations and clinical phenotypes of the patients are listed in Table 1.

The m.3243A > G in the *tRNA^{Leu(UUR)}* (21.3%) and the m.8344A > G in the *tRNA^{Lys}* (33.3%) were the most frequently identified in Korean MELAS and MERRF patients, respectively. However, the m.3243A > G has been identified in up to 80% of MELAS patients, since Goto *et al.* (1990) first reported it. Shoffner *et al.* (1995) also reported 90% of MERRF patients have the m.8344A > G mutation. The low detection frequency of causative mutations might be due that the mutational screen was done by using blood DNA instead of affected muscle DNA.

The m.3243A > G mutation found in 10 MELAS patients showed heteroplasmy in all the patients with a range of 20-91%. In the MT5 family, the proband revealed heteroplasmy of 36%, but her mother and younger brother showed barely 1-2% (Figure 1A). Her father showed no mutation. The proband showed typical MELAS phenotype, including stroke-like episodes, seizures, myopathy, mental retardation, and diabetes. When she was 15 years old, she died due to the cardiorespiratory arrest. However, her other family members have displayed no sign of MELAS symptom. The MT24 patient revealed the highest rate of heteroplasmy
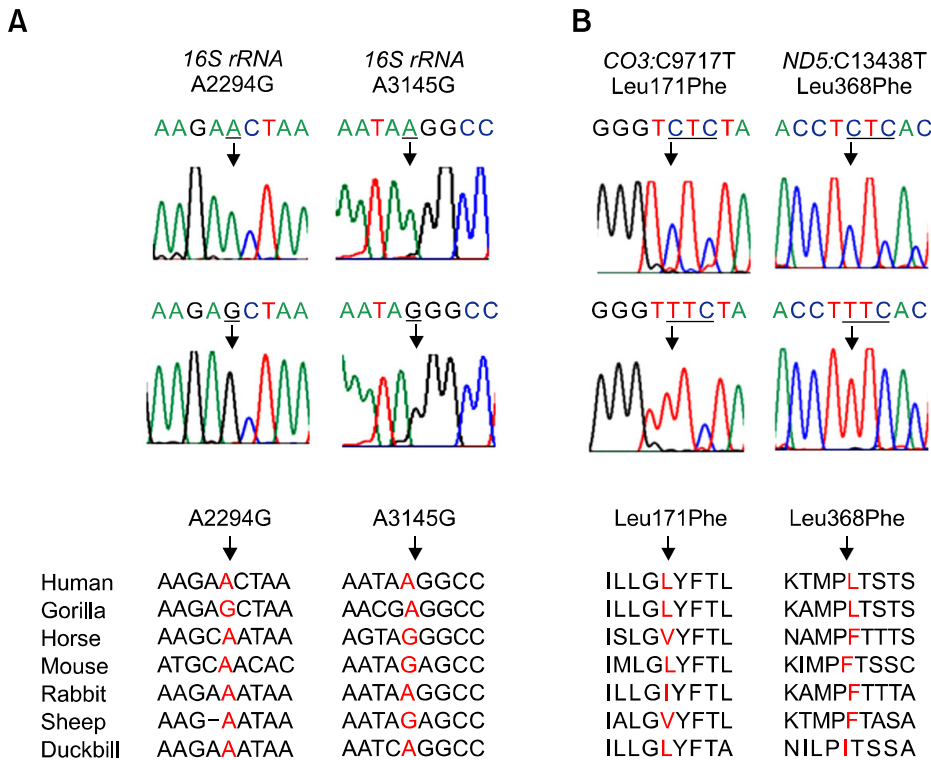
Figure 2. Sequencing chromatograms and their conservation for the unreported mutations. (A) Mutations in the *rRNA* gene. (B) Mutations in the coding genes.

(91%) among the patients that had m.3243A > G in this study. When she was 20 years old, tracheostomy was done due to bilateral phrenic nerve palsy. She showed very severe myopathy, compared to other patients with m.3243A > G mutations. The m.3271T > C mutation was found in a MELAS family (MT4). The heteroplasmic rates were about 41%, 9%, and 45% for the proband, his mother, and elder sister, respectively (Figure 1B). The proband showed generalized tonic-clonic seizures, stroke-like episodes, moderate muscle weakness, and increased level of blood and CSF lactate. However, his elder sister and mother showed only mild muscle weakness. The m.10191T > C in the ND3 was found in a MELAS patient (MT10) with a 94% heteroplasmic rate. However, his brother showed neither mutation (0%) nor MELAS symptom (Figure 1C). This mutation has been previously reported in the epilepsy, stroke, optic atrophy, and cognitive decline (ESOC) and Leigh-like patients (Taylor *et al.*, 2001; McFarland *et al.*, 2004). However, the patient showed neither optic atrophy nor cognitive decline.

The m.8344A > G in the *tRNA*^Lys, which was found in 4 MERRF patients, showed 76% or higher heteroplasmic rate. In the MT1 MERRF family, the proband and his mother showed rates of about 89% and 53%, respectively (Figure 1D). The proband revealed the MERRF phenotypes with myoclonic jerks, generalized tonic-clonic seizures, and myopathy. His mother also revealed MERRF clinical symptoms, except for myopathy. In the MT6 MERRF/PEO overlapping family, the proband revealed about 99% of heteroplasmy. However, his mother showed only about 4%, and his father showed no mutation (Figure 1E). He showed ophthamoplegia, myopathy, epilepsy, and mental retardation. His mother and father were normal in appearance. The m.8363G > A in the *tRNA*^Lys was found in a MERRF patient (MT63) who had symptoms of dysarthria, sensorineural hearing loss, swallowing difficulties, and proximal weakness. In addition, she also had lipomas on the right neck and forearm. The patient showed about 53% of heteroplasmy.

Four unreported mutations were also identified in each different patient: m.2294A > G and m.3145A > G in the *16S rRNA*, m.9717C > T (Leu171Phe) in the *CO3*, and m.13438C > T (Leu368Phe) in the *ND5* (Figure 2). These 4 mutations were not found in 200 controls. Therefore, they might be associated with the corresponding disease (Table 1). The homoplasmic m.9717C > T mutation was identified in a MELAS/PEO overlapping family (MT44). The family showed progressive ophthamoplegia, and also had typical MELAS features, including ragged-red fibers (RRF). The m.13438C > T was identified in a MELAS patient who had proximal

Table 1. Phenotypic characteristics of patients with causative or unreported mtDNA mutations.

Gene	Nt change[a] (AA change)	Patient (Sex)	Main symptom	Age (yrs) Exam	Age (yrs) Onset	Clinical manifestations	Conser[b] (H/M/R/S)	Mutation load (%)[c]	Reference
tRNA^Leu(UUR)	m.3243A > G	MT3 (M)	MELAS	32	21	Dysarthria, tonic-clonic seizure, mild weakness, sensorineural hearing loss, diabetes, RRF	A/A/A/A	21	Goto *et al.*, 1990
	m.3243A > G	MT5 (F)	MELAS	13	Cong-enital	Stroke-like episode, seizure, myopathy, mental retardation, diabetes, died at 15 yrs for cardiorespiratory arrest		36	
	m.3243A > G	MT7 (F)	MELAS	18	15	Lactic acidosis, stroke-like episode, myopathy, generalized tonic-clonic seizure, short stature		40	
	m.3243A > G	MT24 (F)	MELAS	26	20	Myopathy, at 20 years, tracheostomy due to bilateral phrenic nerve palsy		91	
	m.3243A > G	MT30 (M)	MELAS	26	15	Tonic-clonic seizure, sensorineural hearing loss, diplopia, increased blood lactate, hypotonia, ataxia, myopathy		36	
	m.3243A > G	MT33 (M)	MELAS	23	20	Increased blood lactate, myopathy, stroke-like episode		30	
	m.3243A > G	MT34 (M)	MELAS	28	25	Tonic-clonic seizure, increased blood lactate, stroke-like episode, dysarthria, mild mental retardation		20	
	m.3243A > G	MT39 (M)	MELAS	47	42	Stroke-like episode, diabetes, sensorineural hearing loss, dysarthria, increased blood lactate, hypotonia		23	
	m.3243A > G	MT61 (M)	MELAS	13	8	Stroke-like episode, increased blood lactate, mild mental retardation, migraine, short stature, lean body		23	
	m.3243A > G	MT72 (F)	MELAS	29	27	Tonic-clonic seizure, stroke-like episode, increased blood lactate, migraine			
	m.3271T > C	MT4 (M)	MELAS	13	8	Tonic-clonic seizure, stroke-like episode, myopathy and muscle weakness increased blood and CSF lactate	T/T/T/T	41	Goto *et al.*, 1991,
ND3	m.10191T > C (Ser45Pro)	MT10 (M)	MELAS	21	17	Myoclonus epilepsy, generalized tonic-clonic seizure, migraine, mild mental retardation	S/S/G/G	94	Taylor *et al.*, 2001
ND5	m.13438C > T* (Leu368Phe)	MT53 (M)	MELAS	24	21	Muscluar weakness and myopathy, mild mental retardation	L/F/F/F	100	This study
CO3	m.9717C > T* (Leu171Phe)	MT44 (M)	MELAS/ PEO	22	20	Ophthalmoplegia, myopathy, increased blood lactate, RRF	L/L/I/V	100	This study
tRNA^Lys	m.8344A > G	MT1 (M)	MERRF	21	14	Myoclonic jerks and tonic-clonic seizure, muscle weakness, myopathy, cerebellar ataxia	A/A/A/A	89	Shoffner *et al.*, 1990
tRNA^Lys	m.8344A > G	MT2 (M)	MERRF	24	15	Myoclonus epilepsy, muscle weakness, RRF, mild mental retardation		80	
tRNA^Lys	m.8344A > G	MT6 (M)	MERRF/ PEO	25	15	Ophthalmoplegia, myoclonus epilepsy, dysarthria, mild mental retardation, muscle weakness and mild myopathy		99	
tRNA^Lys	m.8344A > G	MT38 (M)	MERRF	20	10	Proximal muscle weakness and myopathy, migraine		6	
tRNA^Lys	m.8363G > A	MT63 (F)	MERRF	53	45	Dysarthria, sensorineural hearing loss, swallowing difficulty, proximal myopathy, lipoma (right neck and forearm), tremor	G/G/G/G	53	Santorelli *et al.*, 1996
16S rRNA	m.3145A > G*	MT13 (M)	MERRF	20	2	Myoclonus epilepsy, cerebellar ataxia, tremor, moderate mental retardation, hypertonia	A/G/G/A	100	This study
16S rRNA	m.2294A > G*	MT60 (M)	MERRF	33	30	Myoclonus epilepsy, sensorineural hearing loss, myopathy, migraine, tremor, short stature	A/A/A/A	100	This study

[a]Asterisks (*) have not been reported in the MITOMAP database (http://www.mitomap.org), [b]Conservation: H, human; M, mouse; R, rabbit; S, sheep; [c]Proband's heteroplasmic rates.

Table 2. Mutations reported as both pathogenic and polymorphic.

Gene	Nt change (AA change)	Family	Conser[a] (H/M/R/S)	Phenotype[b]	Control	Reference
12SrRNA	m.827A > G	MT40, 81, 90	A/A/A/A	Pol/DEAF	1/93	Li *et al.*, 2005
	m.961T > C	MT11, 34	T/A/A/T	Pol/DEAF	3/187	Li *et al.*, 2005
16SrRNA	m.2835C > T	MT53, 77	C/A/T/A	Pol/Rett syndrome	2/96	Cardaioli *et al.*, 1999
ND1	m.3316G > A (Ala4Thr)	MT41	A/M/I/I	Pol/NIDDM, PEO, LHON	3/81	Matsumoto *et al.*, 1999 Lam *et al.*, 2001
	m.3394T > C (Tyr30His)	MT70, 77	Y/Y/Y/Y	Pol/LHON, NIDDM	2/83	Puomila *et al.*, 2007
	m.3497C > T (Ala64Val)	MT6	A/L/L/A	Pol /LHON	2/86	Lam *et al.*, 2001
ND2	m.5460G > A (Ala331Thr)	MT7, 81	A/I/I/V	Pol /AD, PD	9/201	Lin *et al.*, 1992 Schnopp *et al.*, 1996
CO1	m.7119G > A (Asp406Asn)	MT60	D/D/D/N	Pol/MS	0/200	Ban *et al.*, 2008
CO3	m.9438G > A (Gly78Ser)	MT10	G/G/G/G	Pol /LHON	0/200	Oostra *et al.*, 1995
ND4	m.12026A > G (Ile423Val)	MT35, 50, 91	I/M/T/I	Pol /DM	3/99	Mitchell *et al.*, 2006
ND5	m.13708G > A (Ala458Thr)	MT36	A/A/A/L	Pol/LHON, MS	4/85	Yu *et al.*, 2008

[a]H, human; M, mouse; R, rabbit; S, sheep; [b]Pol, polymorphism; DEAF, maternally inherited DEAFness; NIDDM, non-insulin dependent diabetes mellitus; AD, Alzheimer's disease; PD, Parkinson's disease; DM, diabetes mellitus; MS, multiple sclerosis.

myopathy and mental retardation (MT53). The m.3145A > G was identified in a MERRF patient with cerebellar ataxia, tremors, moderate mental retardation, and hypertonia. The m.2294A > G was identified in a MERRF patient (MT60) who also had an additional mutation of m.7119G > A (Asp406Asn) in the *CO1* (Table 2). He showed myoclonus epilepsy, sensorineural hearing loss, and tremors.

Mutations reported as both associative and polymorphic

Eleven mutations that have been previously reported to be both pathogenic (or as risk factor) and polymorphic were identified (Table 2). The m.827A > G, m.961T > C, and m.2835C > T were located on the non-coding genes, and m.3316G > A, m.3394T > C, m.3497C > T, m.5460G > A, m.7119G > A, m.9438G > A, m.12026A > G and m.13708G > A were located on the coding genes. All of these mutations showed homoplasmy. Of them, 9 mutations were also found in the controls with even lower frequencies, but m.7119G > A in the *CO1*, and m.9438G > A in the CO3 were not found in the controls.

The m.961T > C, m.3497C > T, m.5460G > A, and m.9438G > A were found from the patients who also had well confirmed pathogenic mutations. The m.961T > C in the *12S rRNA* was identified from the MT34 MELAS family that also showed the m.3243A > G. The m.961T > C has been reported to be related to both maternally inherited DEAFness

(Li *et al.*, 2005). The m.3497C > T (Ala64Val) in the *ND1* was identified in the MT6 MERRF/PEO family that had the m.8344A > G mutation. This mutation has been reported to be related to LHON (Matsumoto *et al.*, 1999). The m.5460G > A (Ala331Thr) in the *ND2* was identified in the MT7 MELAS patient who also had the m.3243A > G mutation. The m.5460G > A has been reported to be associated with Alzheimer's or Parkinson's patients (Lin *et al.*, 1992; Schnopp *et al.*, 1996). The m.9438G > A (Gly78Ser) in the *CO3* was identified in the MT10 MELAS patient who also had the m.10191T > C mutation. This mutation has been reported to be associated with LHON (Oostra *et al.*, 1995). The m.7119G > A (Asp406Asn) in the *CO1* was found in a MERRF patient (MT60) who also had a suspected mutation m.2294A > G in the 16S rRNA. The m.7119G > A mutation was recently reported in multiple sclerosis patients associated with the haplogroup U (Ban *et al.*, 2008).

Polymorphisms identified by complete mtDNA analysis

Whole mtDNA sequencing analysis (49 patients) identified 40 unreported variations in the MITMAP website (Ruiz-Pesini *et al.*, 2007). The complete sequencing data and corresponding haplobroup of each sample were provided as Supplemental data Table S1. As shown in Table 3, 13 variations were even not reported in the mtDB (http://www.genpat.

Table 3. Unreported mtDNA polymorphisms found in MELAS or MERRF patients.

Gene	Nt change[a]	AA change[b]	Patients	Control	Conservation[c] (H/M/R/S)
tRNA^Phe	m.592C > T	-	MT63	2/184	C/T/C/-
12S rRNA	m.1009C > T*	-	MT87	1/93	C/A/A/A
16S rRNA	m.1766T > C*	-	MT71	1/123	T/A/A/C
	m.2833A > G*	-	MT25	3/155	A/A/A/G
ND1	m.4035A > G	Syn	MT62		L/L/L/L
	m.4129A > G*	Thr275Ala	MT25	4/200	T/A/A/A
ND2	m.4538C > T*	Syn	MT75		S/S/S/S
	m.4926C > T*	Leu153Phe	MT81	2/90	L/I/M/L
	m.5086C > T*	Thr206Ile	MT25	5/210	T/M/I/I
	m.5222A > G*	Syn	MT11		L/L/L/M
CO1	m.6053C > T*	Syn	MT21		N/D/D/D
	m.6086T > C*	Syn	MT55		H/H/H/H
	m.6614T > C*	Syn	MT81		F/F/F/F
	m.6863A > G	Syn	MT44		V/V/V/V
	m.6899G > A*	Syn	MT75		M/I/I/I
CO2	m.8029C > T*	Syn	MT25		A/L/L/M
	m.8065G > A	Syn	MT90		L/L/L/L
ND6L	m.8419T > C*	Syn	MT67		L/I/L/L
ATP6	m.8802T > C	Syn	MT50		F/F/F/F
CO3	m.9362A > G*	Syn	MT25		L/L/L/L
	m.9650C > T	Syn	MT2, 38		H/H/H/H
	m.9977T > C*	Syn	MT46		Y/Y/Y/Y
ND3	m.10295A > G	Syn	MT75		L/I/A/S
ND4	m.10882T > C	Syn	MT41		F/L/L/L
	m.11255T > C*	Tyr166His	MT75	1/95	Y/L/Y/Y
	m.11365T > C*	Syn	MT7		A/A/A/A
	m.11383T > C*	Syn	MT98		P/P/P/P
	m.11389C > T*	Syn	MT33		Y/Y/Y/Y
	m.11854T > C*	Syn	MT70		A/A/T/T/
ND5	m.12396T > C*	Syn	MT87		L/L/V/A
	m.12708C > T	Syn	MT91		F/F/F/F
	m.12952G > A*	Ala206Thr	MT77	1/130	A/N/N/P
	m.13437T > C*	Syn	MT41		P/P/P/P
	m.13602T > C*	Syn	MT24		Y/Y/Y/Y
	m.13819T > C*	Phe495Leu	MT87	1/135	F/V/I/I
ND6	m.14505A > G	Syn	MT62		L/L/L/L
	m.14608C > T	Syn	MT32		K/K/K/K
	m.14629C > T*	Syn	MT39		G/G/G/G
CytB	m.15397A > G	Syn	MT72		K/K/K/K
	m.15565T > C*	Syn	MT77		Y/Y/Y/Y

[a]Asterisks (*) have not been reported in the MITOMAP (http://www.mitomap.org), but referred in the Human Mitochondrial Genome Database (http://www.genpat.uu.se/mtDB), [b]Syn, synonymous mutation; [c]H, human; M, mouse; R, rabbit; S, sheep. MtDNA mutations in MELAS and MERRF patients.

uu.se/mtDB) (Ingman and Gyllensten, 2006). Most variations in the coding genes were synonymous, but six were missense mutations (m.4129A > G (Thr275Ala) in the *ND1*, m.4926C > T (Leu153Phe) and m.5086C > T (Thr206Ile) in the *ND2*, m.11255T > C (Tyr166His) in the *ND4*, m.12952G > A (Ala206Thr) and m.13819T > C (Phe495Leu) in the *ND5*). All the 6 missense mutations and 4 mutations in the non-coding genes exhibited homoplasmy and were also found in controls. Therefore, they were regarded as not the genetic causes of the mitochondrial diseases, but rare polymorphisms.

Pathological findings

Muscle biopsies were performed in 23 patients (MELAS 15, MERRF 6, MELAS/PEO 1, MERRF/PEO 1) of 61 studied patients from the biceps brachii ($n = 21$) or the quadriceps ($n = 2$).

Among them, ragged-red fibers were found in 21 patients (91%). However, electron microscopic examination showed mitochondrial proliferation or enlarged abnormal mitochondria in all patients. In addition, some patients (52%) displayed mitochondria with over abundant or branched cristae.

Discussion

Mitochondrial DNA mutations in 61 patients clinically diagnosed as MELAS or MERRF were examined. The 12 well-confirmed common pathogenic mutations were first screened for all the samples, and then the whole mtDNA sequence was determined in 49 patients. From the analysis, 5 confirmed pathogenic mutations were identified in 17 patients (27.9%), and 4 pathogenically suspected unreported mutations were identified in 4 patients (Table 1). The detection rate of two major pathogenic mutations (A3243G and A8344G) was 22.9%, which is well consistent with the previous Korean study (23.1%) by Kwon et al. (2004). Ten mutations that have been reported as both pathogenic and polymorphic were found in 18 patients (Table 2). Thirteen novel polymorphisms which have not been reported in either of the MITOMAP (http://www.mitomap.org) or the mtDB (http:www.genpat.uu.se/mtDB) were also identified (Ingman and Gyllensten, 2006; Ruiz-Pesini et al., 2007).

Most pathogenic mutations showed heteroplasmy, and the rates were considerably different within the family members (Figure 1). For instance, proband and his sibling of the MELAS family (MT10) showed 94% and 0%, respectively, and proband and his mother of the MERRF/PEO overlapping family (MT6) showed 99% and 4%, respectively. Patients with a higher rate of mutations exhibited a tendency of more severe clinical phenotypes, but not in all cases. The m.3243A>G mutation showed a 20-40% ratio of mutants in most patients, but the MT24 patients showed a very high heteroplasmic rate (91%). This patient showed MELAS symptoms with an additional phenotype of severe respiratory difficulty. Individuals with 1-2% heteroplasmy showed no sign of MELAS symptoms in the MT5 family. The m.8344A>G mutation showed 50-90% ratios in the MERRF patients, but MT6-1 revealed almost homoplasmic mutation (99%). The MT6-1 patient showed the MERRF with an additional phenotype of ophthamoplegia, which is a frequent symptom of PEO. In the MT1 MERRF family, the proband (89%) and his mother (53%) revealed similar MERRF phenotypes. However, the proband showed

epilepsy, but his mother had no symptoms of epilepsy. However, the MT4 MELAS family with m.3271T>C mutation showed a slightly different aspect. The proband (40%) and his mother (9%) showed stroke-like episodes and generalized tonic clonic seizure, but his elder sister (45%) revealed a less severe phenotype with only lactic acidosis. Several studies have suggested the heteroplasmic rates are associated with the severity of clinical phenotypes (Koga et al., 2000; Salpietro et al., 2003), but others have suggested no or weak relation (Harrison et al., 1997; Dobrowolski et al., 2009). It is known that the heteroplasmic rates of pathogenic mutations are depending on the tissue kinds, even if they originated from an individual.

The 4 unreported mutations suspected for genetic defects of mitochondrial diseases were identified as homoplasmy. The m.2294A>G in the 16S rRNA was identified in a MERRF patient (MT60) who also had the m.7119G>A (Asp406Asn) in the CO1, which has been recently suggested as an association with multiple sclerosis as a rare polymorphism (Ban et al., 2008). Both mutations were well conserved in different species and were not found in the 200 Korean controls (Tables 1 and 2), although they have been reported as rare polymorphism (http:www.genpat.uu.se/mtDB). Thus, it is likely that the two combined mutations may function as a genetic risk factor for MERRF. Although the m.3145A>G and m.13438C>T (Leu368Phe) in the MELAS patients (MT53), and m.9717C>T (Leu171Phe) in the MELAS/PEO overlapping patient were not found in 200 controls, the mutation sites were less conserved in different species. Thus, it appears that they might be associated with the disease as indirect risk factors rather than as primary genetic defects. Several secondary mutations (m.4435A>G, m.12192G>A, and m.15951A>G) have been reported to elevate clinical symptoms in the LHON patients (Mimaki et al., 2003; Qu et al., 2006).

This study identified several mutations that have been reported in both pathogenic and polymorphism (Table 3). Since most of them were also found in controls, they might be not pathogenic, but polymorphic in the Korean population. However, the m.9438G>A, which was not found in controls, might function as genetic risk factors or secondary mutations for the mitochondrial diseases. Carelli et al. (2003) suggested a two-locus genetic model that involves a primary mitochondrial mutation and a nuclear modifier. Several decades of polymorphic variants that have been not reported in the MITMAP (Table 3) were also identified. Of them, 13 mutations were not reported, even in the haplogroup studies.

Since reports that LHON patients are preferentially associated with the haplogroup J (Hofmann *et al.*, 1997; Torroni *et al.*, 1997), association studies have become an important approach to uncover the function of mtDNA variations. Although the association study was not performed due to the small size of patient groups, the whole mtDNA sequencing data would be used for further haplogroup studies of Korean mitochondrial patients. Because this study was performed using blood DNA, the suggested rates of causative mutation identification and heteroplasmic loads would be different from the affected muscles. If further mutational study is performed using DNAs from affected tissues, the mutational distribution will be characterized more exactly in Korean MELAS and MERRF patients. This study would be helpful for the molecular diagnosis of mitochondrial diseases, as well as for the basic establishment of the mtDNA database in Korea.

Methods

Patients

This study included 61 independent mitochondrial patients of Korean origin where 43 patients had MELAS, and 18 patients had MERRF (47 isolated cases and 14 families). Of them, 2 patients showed MELAS/PEO (progressive external ophthalmoplegia) overlapping syndrome, and 1 patient demonstrated MERRF/PEO overlapping phenotype. The study also included about 200 healthy controls who had no clinical features and family history of mitochondrial disorders. All participants included in this study provided written informed consent according to the protocol approved by the Ethics committee of Ewha Woman's University, School of Medicine.

DNA isolation

Total DNA was extracted from the whole peripheral blood samples by using the QIAamp Blood DNA mini kit (Qiagen, Hilden, Germany). The paternity and maternity were confirmed in familial samples by the genotyping of 15 microsatellite markers that were provided in the PowerPlex 16 kit (Promega, Madison, WI).

PCR amplification and sequence analysis

Six primer pairs were designed to detect 12 common mutations (m.3243A$>$G, m.3291T$>$C, m.3460G$>$A, m.3271T$>$C, m.8344A$>$G, m.8356T$>$C, m.8363G$>$A, m.8993T$>$G/C, m.10158T$>$C, m.10191T$>$C, m.11778G$>$A and m.14484T$>$C). The primer sequences and PCR conditions used are available on request to corresponding author. The entire mitochondrial genome was sequenced by the PCR by using 46 primer sets of the MitoSEQr resequencing system (Applied Biosystems, Foster city, CA). The PCR amplification condition consisted of initial denaturation at 96°C for 5 min, followed by 35 cycles at 94°C for 30 s, 60°C for 45 s, 72°C for 45 s, and a final extension at 72°C for 10 min. PCR products were purified by the treatment of exonuclease I-shrimp alkaline phosphatase (Fermentas, Canada) and sequenced by an automatic genetic analyzer ABI3100 using the big dye terminator cycle sequencing ready reaction kit (Applied Biosystems). Sequence variations were confirmed by analyzing both strands of DNA. The sequences were compared with the revised Cambridge reference sequence (NC_012920) by using the SeqScape software (Ver. 2.1, Applied Biosystems) (Andrews *et al.*, 1999).

Determination of heteroplasmy

The heteroplasmic rates of the mtDNA mutations were determined by screening bacterial clones that had the corresponding mtDNA fragment (Uusimaa *et al.*, 2004). The DNA fragments, obtained by PCR amplification, were subcloned into the pGEM-T Easy vector (Promega), which was then used to transform *E. coli* (DH5α). Plasmid DNA was isolated from 100 colonies/mutation by using a plasmid DNA isolation kit (Intron Biotechnol., Korea), and the mutation was determined by the sequencing of the insert DNA. The rate of heteroplasmy was measured by the counting of clones that had a mutant sequence.

Clinical and pathological assessments

Careful clinical information and neurological examinations were obtained, and the MELAS and MERRF were diagnosed to meet the clinical criteria (Bernier *et al.*, 2002). All patients underwent brain MRIs and electrophysiological studies. Whole brains were scanned by using a slice thickness of 7 mm and a 2-mm interslice gap to produce 16 axial images (Siemens Vision: Siemens, Germany). Muscle biopsies were done under local anesthesia, and cross-sections of the biopsy tissue were stained with hematoxylin-eosin, modified Gomori-trichrome, cytochrome c oxidase, and succinate dehydrogenase. For the electron microscopic observation, the specimen was fixed in 2% glutaraldehyde in 25 mM cacodylate buffer at pH 7.4, and processed for semithin and ultrathin studies.

Supplemental data

Supplemental Data include a table and can be found with this article online at http://e-emm.or.kr/article/article_files/SP-42-6-05.pdf.

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